Comet Assay Demonstrates a Higher Ultraviolet B Sensitivity to DNA Damage in Dysplastic Nevus Cells Than in Common Melanocytic Nevus Cells and Foreskin Melanocytes

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We used the single cell gel electrophoresis assay (comet assay) to study ultraviolet B (UVB)-induced DNA damage in pigment cells. This assay detects DNA damage, mainly DNA strand breaks and alkali labile sites in the DNA molecule. We studied the effect of biologically relevant doses (comparable to 2–3 MED (minimal erythemal dose) for *in vivo* irradiated full-thickness skin) of monochromatic UVB light of 302 nm on cultured melanocytes derived from foreskin, common melanocytic nevi, and dysplastic nevi. We were able to demonstrate a linear dose-response relationship between UV dose and the migration coefficient of the comet tail in all three types of pigment cells. Nevus cells originating from dysplastic nevi showed the highest sensitivity to UVB irradiation: 65% higher induction of DNA damage compared to the induction in foreskin melanocytes. Common melanocytic nevus cells were most resistant and showed a 30% lower induction of DNA damage in comparison to foreskin melanocytes. Differences in chromatin structure and cell cycle profile may influence the results of the comet assay. Control experiments with x-ray irradiation, which is well known to produce direct DNA strand breaks via radical formation, revealed only small differences between the three types of melanocytic cells. It is unlikely, therefore, that intrinsic nuclear characteristics may account for the observed differences. *J Invest Dermatol 106:1198–1202, 1996*

The increase in the incidence of cutaneous melanoma is alarmingly high compared to that of other malignancies, especially in young adults (Sober *et al*, 1991). It has been reported that between 0.5 and 12% of melanomas occur in individuals with the familial atypical multiple mole and melanoma (FAMMM) syndrome, which is a hereditary disorder characterized by multiple clinically atypical (dysplastic) nevi and a high risk of melanoma. Melanomas are more often diagnosed in family members with a sun-sensitive type I skin. This indicates a role for environmental factors such as sun exposure in the induction of melanoma in addition to genetic predisposition (Bergman *et al*, 1986). Epidemiological studies show contradictory findings with respect to the relationship of solar irradiation and the incidence of melanoma (Koh *et al*, 1990).

In recent years, studies have been performed with fibroblasts of FAMMM patients to evaluate their genetic susceptibility to some effects of ultraviolet light. Some authors found an enhanced sensitivity to UV in these patients with respect to survival (Smith *et al*, 1982) or sister chromatid exchange (Roser *et al*, 1989; Hürlimann *et al*, 1992). Other groups were not able to confirm these results (Jaspers *et al*, 1987; Thielmann *et al*, 1991).

Few studies have been performed with respect to the effect of UV irradiation on the obvious target cells of cutaneous melanoma: melanocytes derived from dysplastic nevi (DN), which are the well known precursor lesions of the superficial spreading type of cutaneous melanoma (Kraemer *et al*, 1983). In an earlier study, we have demonstrated a lower induction of cyclobutane thymine dimers by ultraviolet B (UVB) light of 302 nm in DN cells compared to foreskin-derived melanocytes (FS-mc) and common melanocytic nevus (CMN) cells (Noz *et al*, 1994). Nonetheless, it is possible that different types of UV-induced (DNA) damage may be more prominent in DN cells than in other pigment cells. The prominent feature of pigment cells is their pigment-producing capacity. The interaction of melanin and UV radiation may lead to oxidative DNA damage or DNA strand breaks.

To date, the single cell gel electrophoresis assay, also called the comet assay (Singh *et al*, 1988), has been used to evaluate DNA strand breaks and alkali labile sites that result from x-ray radiation and chemical agents in HeLa cells and different human cell types. Even with a limited number of cells it has proved to be a sensitive and reproducible assay (McKelvey-Martin *et al*, 1993). Because dysplastic nevus cells are cultured with great difficulty, the comet

Manuscript received May 2, 1994; revised January 23, 1996; accepted for publication February 7, 1996.

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Abbreviations: FAMMM, Familial atypical multiple mole and melanoma syndrome; CMN, common melanocytic nevus; DN, dysplastic nevus; FS-mc, foreskin-derived melanocyte; PBS, phosphate-buffered saline.

0022-202X/96/$10.50

assay is pre-eminently suited for the evaluation of DNA damage in these cells. The assay has also been used to demonstrate the indirectly induced strand breaks that occur during excision repair (e.g., of UVC-induced thymine dimers) (Gedik *et al*, 1992; Green *et al*, 1992; Arlett *et al*, 1993).

The aim of this study was to evaluate UV-induced DNA damage other than pyrimidine dimers. We used the comet assay to measure immediately induced DNA damage, mainly DNA strand breaks and alkali labile sites, in cultured pigment cells derived from DN, CMN, and foreskin after physiological relevant doses of UVB.

MATERIALS AND METHODS

Culture of Human Melanocytes from Foreskin, Nevocellular, and Dysplastic Nevi Human (nevo)melanocyte cultures were established from neonatal foreskin (n = 5), CMN (n = 5), and DN (n = 4) in a culture system that has been described in detail previously (Noz *et al*, 1994). Foreskins were derived from circumcision surgery. All CMN were acquired from patients without DN and without a personal or family history of melanoma. The DN were obtained by excision biopsy from two patients with FAMMM syndrome and two patients with sporadic (i.e., nonfamilial) dysplastic nevus syndrome. Part of the nevus was used for histological evaluation; all DN used in the experiments showed moderate atypia (on the increasing scale of slight, moderate, and severe atypia). Experiments were performed with the pigment cells in their third to ninth passage. Prior to irradiation the cells were carefully trypsinized with 0.01% trypsin with 0.02% ethylenediammine tetraacetic acid and 0.1% glucose in phosphate-buffered saline (PBS), pH 7.3.

UV Light Source, UV Dosimetry, and Irradiation The irradiation was performed with a 1000-W mercury-xenon arc lamp source with a narrow band interference filter of 302-nm wavelength ± 4.0 half-band-width (Oriel Corp., Stamford, CT). Dosimetry was performed with a galvanometer AL4-Microva in conjunction with a thermopile CA1 plus infrasil window (Kipp & Zonen, Delft, The Netherlands); the dose rate ranged between 4.63 and 5.11 W/m^2. Cells were irradiated in suspension in cold PBS supplemented with 0.1% glucose. The doses ranged between 0 and 200 J/m^2, which is comparable with doses that melanocytes receive when full-thickness skin is irradiated with doses in the range between 0 to 2–3 MED (minimal erythemal dose) (Noz *et al*, 1994). Cells were kept on ice before and immediately after irradiation. The temperature was kept to 4°C till lysis of the cells was complete in order to avoid the possibility of DNA repair.

Ionizing Radiation Cells were irradiated in suspension (cold PBS supplemented with 0.1% glucose) with a Philips Müller RT100 x-ray unit (Eindhoven, The Netherlands) operating at 100 kV and 8 mA, and with a 0.78-mm aluminum filter and a dose rate of 2 Gy/min. Before and immediately after irradiation, the cell suspensions were kept on ice.

Single Cell Gel Electrophoresis (Comet Assay) Single cell gel electrophoresis was performed by a slight adaptation of the method by Singh *et al* (1988). Melanocytes were embedded in agarose gels on frosted microscope slides (Labcraft; Curtin Matheson Scientific Inc., Houston, TX). The cells were lysed for 1 h at 4°C in lysis solution, which consisted of 2.5 M NaCl, 100 mM ethylenediammine tetraacetic acid, 10 mM Tris, 0.3 M NaOH, 1% sodium sarcosinate (Sigma Chemical Co., St. Louis, MO), 1% Triton X-100, and 10% dimethylsulfoxide. The slides were put in a solution of 0.3 N NaOH and 1 mM ethylenediammine tetraacetic acid for 40 min at room temperature without electrical current to allow unwinding of the DNA. Electrophoresis was carried out in the same buffer for 25 min at 25 V and 300 mA. Under these conditions, undamaged DNA will remain in the nucleus. DNA containing strand breaks (alkali-labile sites) will stream toward the anode, and when the DNA is stained with ethidium bromide (2 μg/ml in PBS), this yields the image of a tail of a comet **(Fig 1)**. After putting coverslips on top of the gels, we kept the slides in the dark in a moist room at 4°C until image analysis.

Image Analysis System Description The analysis system is composed of an Aristoplan fluorescence microscope (Leica, Wetzlar, Germany) equipped with a KAF-1400-cooled CCD camera (Photometrics, Tucson, AZ). The chip allows 1348 × 1035 pixels with a pixel size of 6.8 × 6.8 μm and has a Metachrome coating to improve the sensitivity for light of shorter wavelengths (e.g., UV and blue light). The camera head is Peltier-cooled to approximately −40°C to minimize dark current and allow for longer integration times. The VME200A camera controller digitizes the images with a resolution of 12 bits and transfers the pixels at a rate of 500 kHz into a Sparc-330 workstation (Sun, Mountain View, NY). This workstation is equipped with 32 megabytes of memory, a 327-megabyte disk, and a 19-inch color monitor. The analysis of the digitized images of the comets is performed using the TCL-image software package (Multihouse, Amsterdam, The Netherlands).

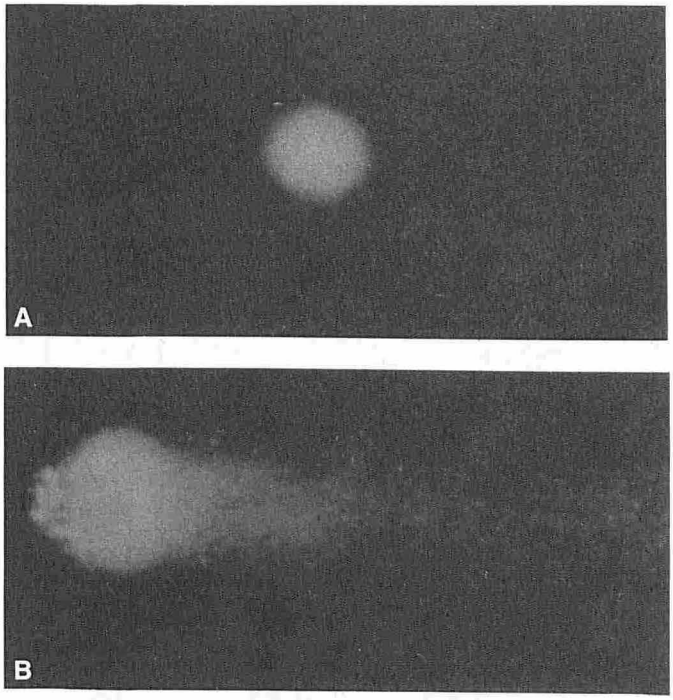

Figure 1. Comet assay shows migration of DNA outside the nucleus after UVB irradiation. *A*, image of the nucleus of a nonirradiated foreskin-derived melanocyte in the comet assay. *B*, image of a nucleus of an irradiated foreskin-derived melanocyte in the comet assay. Nuclei were stained with ethidium bromide.

Image Analysis The comets are digitized in such a way that the direction of the tails coincides with the horizontal axis of the image. The analysis of the individual nuclei is completely automated. The gray-value histogram of the image is determined, and a threshold value is calculated on the basis of the position and the width of the background peak. Thereafter the DNA profile along the comet is determined by integrating the fluorescence, corrected for the background, perpendicular to the direction of the tail of the comet. The shape of the left half of the nucleus (and thus also of the profile) is used as a model for the right half. By mirroring of the front slope of the profile and subtracting this part from the original profile, the comet is split up into a head and a tail part. The total fluorescence of the comet and the fraction of fluorescence that is located in the tail are calculated on the basis of these profiles. As a measure for the migration of the amount of DNA outside the nucleus, the first order moment is determined according to the following formula:

$$M = \frac{1}{L \cdot T} \sum_{x} x \cdot D(x)$$

where M is the migration coefficient, L is the total length of the comet (in pixels), T is the total fluorescence of the comet, x is the distance from the center of the nucleus along the comet (in pixels), and D(x) is the integrated fluorescence as a function of the distance.

Twenty nuclei per sample were analyzed.

Statistical Analysis After scanning a population of 20 cells, we calculated the mean and the standard error of the mean (SEM) of the migration coefficient values for each dose. For each (nevo-)melanocyte culture, two to three independent experiments were performed. Linear regression lines were calculated by processing these separately performed experiments by the method of least squares. The slopes of two regression lines were compared by computing the difference of the slopes of the two lines divided by the standard error of this difference; this quotient yields a value within a standard normal deviate (Noz *et al*, 1994). Differences between unirradiated cells of the three types of pigment cells were evaluated by Student's t tests.

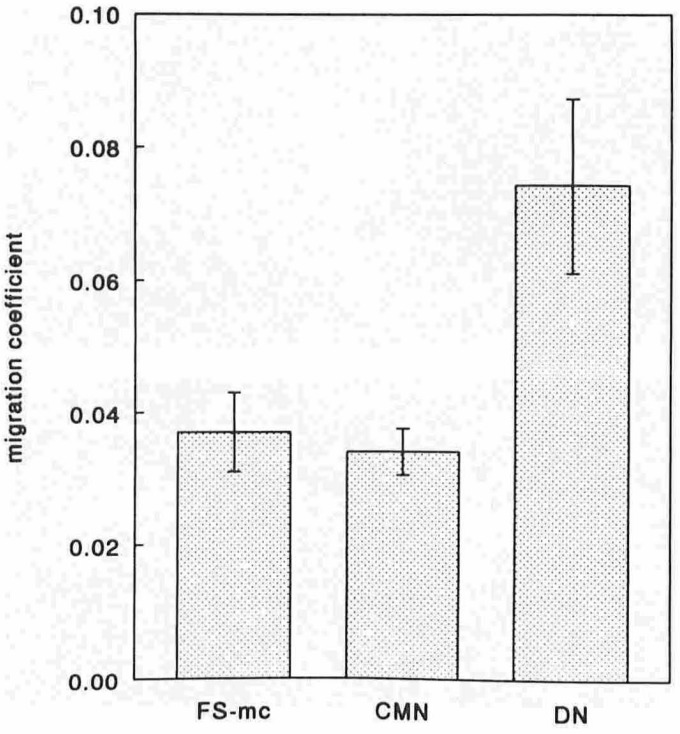

Figure 2. Nonirradiated nuclei of DN show higher migration coefficients than nonirradiated nuclei from FS-mc and CMN. Mean and SEM of the migration coefficients of nonirradiated melanocytes derived from neonatal foreskin (0.037 ± 0.006 arbitrary units (a.u.)), from common melanocytic nevi (0.034 ± 0.003 a.u.), and from dysplastic nevi (0.074 ± 0.013 a.u.). Dysplastic nevus cells differed significantly from foreskin-derived melanocytes ($p < 0.01$) and from common nevus cells ($p < 0.003$).

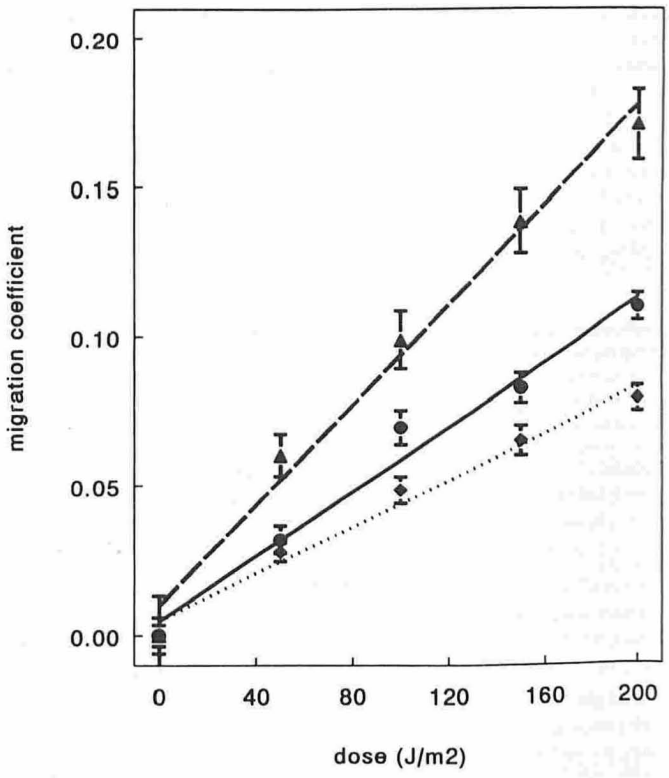

Figure 3. High induction of DNA damage due to UVB irradiation of 302 nm, while CMN cells are least susceptible. Melanocytes derived from foreskins, CMN, and DN were irradiated in suspension in cold PBS with different doses of 302-nm UVB light and immediately subjected to the comet assay. Damaged DNA migrates out of the nucleus. The amount of damage is expressed in the migration coefficient (arbitrary units). This figure shows the comparison of the regression lines representing the linear dose-response relationships between UV irradiation and the migration coefficient in the single cell gel electrophoresis assay in cultured melanocytes derived from neonatal foreskin (●), common melanocytic nevi (◆), and dysplastic nevi (▲). Each point represents the mean of 8–10 separately performed assays. *Error bars*, SEM.

RESULTS

Migration Coefficient of Unirradiated (Nevo-)melanocytes In unirradiated cells, one would expect a low level of DNA damage. In the ideal situation, the migration coefficient would equal zero. **Figure 2** shows the means and SEM of the migration coefficients of the unirradiated pigment cells. Unirradiated DN cells show a significantly higher migration coefficient than unirradiated CMN cells ($p < 0.003$) and unirradiated foreskin-derived melanocytes (FS-mc) ($p < 0.01$). Unirradiated cells from CMN do not differ from unirradiated FS-mc ($p = 0.30$). This implies a higher level of pre-existent DNA damage in nevocytes derived from DN.

Intra- and Interindividual Variation We used the comet assay to measure UVB-induced DNA damage in melanocytes from different sources. For each dose-response experiment, the background value of the unirradiated cells was subtracted from the measured values, and a regression line was calculated for each assay. The correlation coefficient of the regression lines exceeded 0.95. To evaluate the reproducibility of the comet assay, we performed two separate assays with each melanocyte culture and calculated the coefficient of variation of the slopes of the regression lines.

For the experiments performed with UV light, the intraindividual variation of the slopes ranged between 2.1 and 6.8% with the exception of one CMN, which showed a variation of 15.7%. The interindividual variation for FS-mc, CMN, and DN cells was evaluated in UV-treated cultures of four or five different donors per group; the standard errors of the slopes were respectively 8.7, 6.6, and 6.8% of the mean.

For the experiments performed with x-rays, the intraindividual variation ranged between 1.1 and 5.2%, while interindividual variation between two different cultures of each cell type was only 0.9% for FS-mc and 0.2% for DN cells, but 19% for the CMN.

Differences in UVB Susceptibility in the Three Types of Pigment Cells For FS-mc, CMN, and DN cells linear dose-response curves were demonstrated for increase in migration coefficient. The correlation coefficients of the mean linear regression lines for the three types of melanocytes exceeded 0.98, and the coefficient of variation was between 7 and 11%.

The slope of the dose-response curve of DN nevocytes was much steeper than for the other two types of melanocytes **(Fig 3 and Table I)**. In DN cultures we found a 65% higher induction of DNA damage than in FS-mc and a 2.4-fold higher induction than in CMN cells. The regression line of DN cells differed significantly from those of FS-mc ($p << 0.0001$) and of CMN cells ($p << 0.0001$). The slopes of the dose-response curves of FS-mc and CMN cells differed also ($p < 0.001$). Compared to FS-mc we found a 30% lower induction in CMN cells.

X-Ray–Induced DNA Damage in the Three Different Types of Melanocytes The setup of the UV irradiation experiments of the pigment cells in this study was designed to prevent excision repair of DNA base damage. So in these experiments we observed immediately induced DNA strand breaks. Ionizing radiation is known to induce strand breaks directly in the DNA. This has also been evaluated with the comet assay by other investigators. From these studies it became apparent that several factors, such as the different cell cycle stages, chromatin structure, or tight packing of DNA, may influence the migration of DNA in the comet assay. This has been demonstrated for formation of double strand breaks

Table 1. Slopes of the Regression Lines Representing the Exposure-Response Relationship of DNA Damage Measured by Comet Assay in Three Types of Melanocytes upon Exposure to UVB of 302 nm and x-Ray Irradiation[a]

Origin	UV-B, Slope ± SD (10^{-4} U m^2/J)	X-Rays, Slope ± SD (10^{-2} U/Gy)
Foreskin	5.39 ± 0.46	2.40 ± 0.24
Common nevus	3.89 ± 0.32	2.17 ± 0.26
Dysplastic nevus	8.40 ± 0.58	2.07 ± 0.16

[a] Melanocytes derived from foreskins, common melanocytic nevi, and dysplastic nevi were irradiated with monochromatic UVB light of 302 nm and x-rays. After irradiation with different doses, they were immediately subjected to the comet assay (see *Materials and Methods*). The migration coefficient is a measure for DNA damage (strand breaks and alkali labile sites) and is expressed in arbitrary units. Each melanocyte culture showed a linear dose-response relationship between the irradiation dose and the migration coefficient. The slopes of these dose-response curves are calculated from data obtained from melanocytes obtained from five different foreskins, five CMN, and four DN. We performed two separate identical experiments with each melanocyte culture.

(Olive *et al*, 1991, 1992; Elia and Bradley, 1992; Warters and Lyons, 1992), which are measured when the comet assay is performed under neutral conditions. When the assay is carried out under alkaline conditions, as in our experiments, no differences should be observed when cells in different cell cycle stages are used (McKelvey-Martin *et al*, 1993). To exclude intrinsic nuclear characteristics as a cause of the differences between the pigment cells of the three different sources, we have performed control experiments with the x-ray–irradiated cells from the same cultures.

The results for x-ray irradiation are summarized in **Table I** and **Figure 4**. The migration coefficient showed linear dose-response relationships for all three types of melanocytes. The correlation coefficients exceeded 0.98. The coefficients of variation of the slope were between 7.5 and 12%. There was no statistical difference in slope between CMN and DN cells ($p > 0.4$). Between FS-mc and CMN cells, there was a statistically significant difference ($p = 0.02$), while the FS-mc differed slightly from DN cells without reaching significance ($p = 0.07$). The FS-mc were most susceptible for x-ray–induced DNA damage; DN and CMN cells showed a 10% lower induction compared to FS-mc.

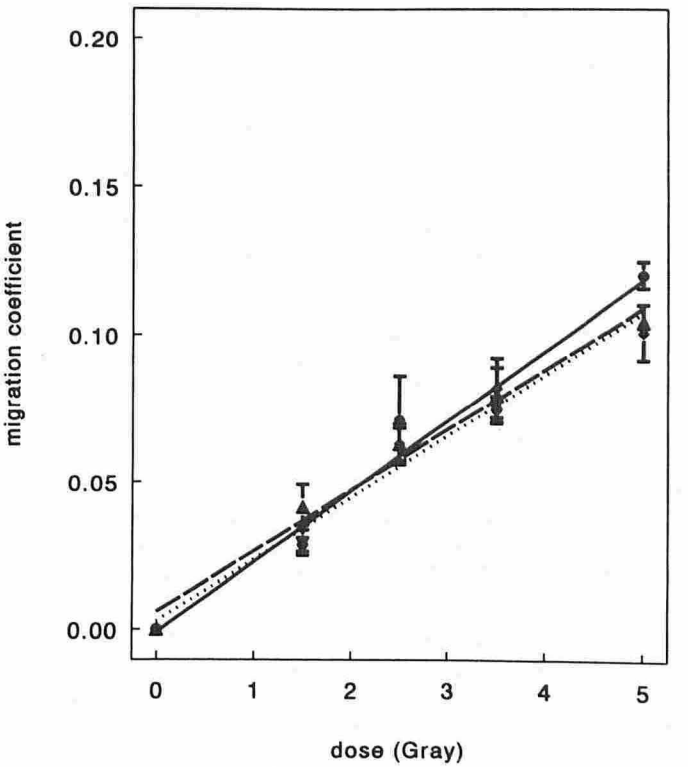

Figure 4. Comet assay shows no differences in susceptibility to DNA damage due to x-ray irradiation between FS-mc, CMN, and DN cells. Comparison of the regression lines representing the dose-response relationships between x-ray irradiation and the migration coefficient in the single cell gel electrophoresis assay in cultured melanocytes from neonatal foreskin (●), common melanocytic nevi (◆), and dysplastic nevi (▲) is shown. Each point represents the mean of four separately performed assays. *Error bars*, SEM.

DISCUSSION

In this study we have shown that the comet assay can demonstrate immediately induced DNA breakage in cultured human melanocytes due to doses of UVB light in a physiologically relevant range. The maximum dose is representative of the amount of UVB that melanocytes can receive when full-thickness skin is irradiated with 2 to 3 MED (Noz *et al*, 1994). We found significant differences in susceptibility to UVB-induced DNA damage between the three types of pigment cells with the comet assay. DN cells were most susceptible, while CMN cells were most resistant to UVB irradiation.

Control experiments with x-ray radiation were performed to rule out the possibility that the observed differences could be a result of certain cellular or nuclear characteristics. They revealed no differences in the induction of DNA damage between the two types of nevus cells. On the other hand, there seemed to be a difference in the x-ray–induced levels of DNA damage between FS-mc and the two types of nevus cells. The FS-mc were slightly more susceptible to x-ray–induced DNA damage. In a previous study, we performed a cell cycle analysis of the three different types of melanocytes and found shifts to the left of the G1 and G2 peaks of the profile of nuclei originating from a DN culture compared to FS-mc and CMN cells (Noz *et al*, 1994). This indicates a more compact chromatin structure of the DNA in DN cells and may explain the lower level of x-ray-induced DNA damage in DN cells compared to FS-mc in the comet assay. If tight packing of DNA is important, then the high levels of UVB-induced DNA damage in DN measured in the comet assay are even more striking.

DNA strand breaks are the main type of DNA lesion following UVA exposure, while pyrimidine dimers predominate with UV of shorter wavelengths (UVB and UVC). Several groups demonstrated the induction of DNA single strand breaks in Chinese hamster V-79 cells and human skin fibroblasts following UVB and UVC exposure by the alkaline elution method, but the strand breaks were detected solely after exposure to high doses of UV light with a low percentage of survival of the cells (Rosenstein, 1988; Lai and Rosenstein, 1990; Matsumoto *et al*, 1991). The maximum dose that the pigment cells received in our study was 200 J/m^2 of 302-nm light. At this dose, a survival of 100% (shoulder area of the survival curve) was found in cultured human melanocytes derived from foreskin (De Leeuw *et al*, 1994). Assuming that the comet assay detects mainly DNA strand breaks, it is interesting that we have detected these DNA lesions after low doses of UV light of short wavelength.

The comet assay has been employed to evaluate directly induced DNA strand breakage resulting from x-rays and several chemicals, e.g., bleomycin. It has also been used to evaluate UV-induced DNA damage, but with the comet assay it has never been possible to demonstrate immediately induced DNA damage after UV irradiation, only the indirect (delayed)-induced DNA strand breaks resulting from DNA repair in the postirradiation incubation period. Therefore, the comet assay has been employed as an indicator for excisable DNA damage (Arlett *et al*, 1993). In contrast to comet assay studies by other investigators with other human cell types, we have demonstrated immediately induced DNA damage in melanocytic cells. Arlett *et al* (1993) have shown that different types of cells

may differ in susceptibility to UV-induced DNA damage in the comet assay. Another interesting finding in our study was the observed difference in the migration coefficients of unirradiated melanocytic cells from the three different sources, suggesting pre-existing DNA damage in DN cells.

We hypothesize that the observed differences in the level of UVB-induced DNA damage in the comet assay may be attributed to the presence and photoreactivity of melanin within the cells under study. Another argument in favor of this hypothesis may be found in similar pilot experiments that we have performed with fibroblasts (non–pigment-containing cells), in which more variable results were obtained (results not shown). A clear increase in migration coefficient was present in fibroblasts at the lowest dose of 50 J/m^2, followed by a leveling off at higher exposure levels. Melanins are highly reactive compounds that are well known to scavenge and produce radicals after UV irradiation (Sarna *et al*, 1985). These radicals may interact with the nucleotides of the DNA molecule and produce DNA strand breakage (Koch and Chedekel, 1986). In our x-ray experiments, no obvious differences were observed in the induction of DNA damage between the three pigment cell types, while the UVB experiments showed marked differences in susceptibility. This also suggests a possible role for photoreactive properties of melanins.

In the comet assay, DN cells are significantly more susceptible to the induction of DNA damage by UVB light than are CMN cells and FS-mc. We found that nonirradiated DN cells may have a higher level of pre-existent DNA damage than other pigment cells. The exact nature of this type of DNA damage will require further investigation.

We thank Ms. I. M. Zandman and K. Schöppink for technical assistance. This work was financially supported by grants from the Netherlands Organization for Scientific Research (NWO) to the Department of Dermatology, University Hospital Leiden (Grant 901-01-126) and to the Department of Cytochemistry and Cytometry, State University of Leiden (Grant 90-129.90) and by the IRS–J.A. Cohen Institute Interuniversity Research Institute for Radiopathology and Radiation Protection, The Netherlands.

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